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Circulation Research (2002), 90(12), 1234-1236

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Isolation and Chromosomal Localization of the Human Endothelial Nitric Oxide Synthase (NOS3) Gene

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Nitric oxide (NO) is an important intercellular signaling molecule synthesized in diverse human tissues by proteins encoded by a family of NO synthase (NOS) genes. The similarity of sequence and cofactor binding sites has suggested that the NOS genes may also be related to cytochrome P450 reductase, as well as to plant and bacterial oxidoreductases. Endothelial NOS activity is a major determinant of vascular tone and blood pressure, and in several important (and sometimes hereditary) disease states, such as hypertension, diabetes, and atherosclerosis, the endothelial NO signaling system appears to be abnormal. To explore the relationship of the endothelial NOS gene to other similar genes, and to delineate the genetic factors involved in regulating endothelial NOS activity, we isolated the human gene encoding the endothelial NOS. Genomic clones containing the 5′ end of this gene were identified in a human genomic library by applying a polymerase chain reaction (PCR)-based approach. Identification of the human gene for endothelial NOS (NOS3) was confirmed by nucleotide sequence analysis of the first coding exon, which was found to be identical to its cognate cDNA. The NOS3 gene spans at least 20 kb and appears to contain multiple introns. The transcription start site and promoter region of the NOS3 gene were identified by primer extension and ribonuclease protection assays. Sequencing of the putative promoter revealed consensus sequences for the shear stress-response element, as well as cytokine-responsive *cis* regulatory sequences, both possibly important to the roles played by NOS3 in the normal and the diseased cardiovascular system. We also mapped the chromosomal location of the NOS3 gene. First, a chromosomal panel of human–rodent somatic cell hybrids was screened using PCR with oligonucleotide primers derived from the NOS3 genomic clone. The specificity of the amplified PCR product was confirmed by human and hamster genomic DNA controls, as well as by Southern blot analysis, using the NOS3 cDNA as probe. Definitive chromosomal assignment of the NOS3 gene to human chromosome 7 was based upon 0% discordancy; fluorescence *in situ* hybridization sublocalized the NOS3 gene to 7q36. The identification and characterization of the NOS3 gene may lead to further insights into heritable disease states associated with this gene product. © 1994 Academic Press, Inc.

INTRODUCTION

Nitric oxide (NO), an unusual and ubiquitous intercellular messenger molecule, subserves diverse functions in a variety of human tissues, including vasodilatation, cytotoxicity, and neurotransmission (reviewed in Moncada *et al.*, 1991; Lowenstein and Snyder, 1992; Nathan, 1992; Marletta, 1993). NO production is catalyzed by a family of nitric oxide synthases (NOS), including at least three genetically distinct isoforms originally isolated from brain, macrophage, and endothelium. NOS isoforms in diverse tissues appear to share a similar overall enzymatic mechanism, involving the oxidation of the terminal guanidino nitrogen of the amino acid L-arginine via a complex reaction involving multiple redox cofactors to yield NO plus citrulline (for review, see Marletta, 1993).

The brain and endothelial NOS isoforms are constitutively expressed in their respective tissues, and both are regulated by intracellular calcium. By contrast, the macrophage isoform is expressed only in response to inflammatory mediators and appears to be calcium-independent. The brain (Bredt *et al.*, 1991) and endothelial (Lamas *et al.*, 1992; Janssens *et al.*, 1992) NOS isoforms also share 60% amino acid sequence similarity but are the products of distinct genes (Lamas *et al.*, 1992). Macrophage NO synthase bears only 50% amino acid sequence similarity to the other isozymes (Lowenstein *et al.*, 1992; Lyons *et al.*, 1992; Xie *et al.*, 1992) and appears to be the product of a third distinct gene. These enzymes are also related to the mammalian cytochrome P450 reductase (Bredt *et al.*, 1991), which has ~30% amino acid sequence identity with the C-terminal half of each of the NOS isoforms cloned to date. The C-terminal half of the

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NO synthases also shows significant amino acid sequence similarity to plant (Karplus *et al.*, 1991) and bacterial oxidoreductases (Ostrowski *et al.*, 1989).

The human brain and macrophage NOS genes have recently been mapped to chromosomes 12 (Kishimoto *et al.*, 1992) and 17 (J. Mudgett, Merck Laboratories, pers. comm.), respectively; thus, the gene family as a whole is not clustered in a single region of the genome. Synteny of the brain and endothelial isoforms, which share similar regulatory mechanisms and greater sequence similarity, is an interesting but untested possibility. The position of the NOS3 gene relative to that of cytochrome P450 reductase, which has been mapped to chromosome 7 (Shephard *et al.*, 1989; Yamano *et al.*, 1990), is also of interest given the similarities between this protein and the C-terminus of the NO synthases. While the N-terminal halves of the NO synthases also show significant sequence conservation, they resemble no other known proteins. However, based upon the presumptive binding of heme in this region of the protein, with a characteristic P450 spectral pattern (reviewed in Marletta, 1993), it has been suggested that the gene for NOS (or a precursor) may have resulted from the fusion of two genes, one encoding a cytochrome P450 and one related to cytochrome P450 reductase (Lowenstein and Snyder, 1992). Mapping the chromosomal location of the NOS3 gene may help clarify the phylogenetic relationships between these genes.

Localization and characterization of the NOS3 gene are also of interest because of its potential role in the pathophysiology of a number of heritable cardiovascular diseases. NO is produced by endothelial cells in response to a number of extracellular signals, including bradykinin, ADP, and thrombin (reviewed in Furchgott and Vanhoutte, 1989). NO production is also increased in endothelial cells exposed to hemodynamic shear stress. The endothelial cell-derived NO acts upon the soluble guanylate cyclase of neighboring smooth muscle cells to effect their relaxation and also appears to inhibit platelet aggregation and adhesion. Levels of NOS activity may also be determined by factors hypothesized to alter NOS3 expression, such as shear stress and cytokines (Lamas *et al.*, 1992; Nishida *et al.*, 1992). Aberrant endothelial NO production and metabolism have been implicated in several important (and in some cases familial) cardiovascular disorders, including hypertension, atherosclerotic heart disease, and diabetes. Identification and characterization of the NOS3 gene should facilitate investigation, e.g., by linkage studies, of possible alterations in the NOS signaling pathway occurring in these disorders.

MATERIALS AND METHODS

Reagents. A human placenta genomic DNA library in bacteriophage λFIX II, pBluescript plasmid and an *in vitro* transcription kit were obtained from Stratagene. The random hexamer-primed DNA labeling kit was from Boehringer Mannheim. Restriction endonucleases and T4 polynucleotide kinase were from New England Biolabs. The Sequenase II sequencing kit, RNase T1, and RNase A were from U. S. Biochemicals. All radionucleotides were purchased from DuPont New England Nuclear. *Taq* DNA polymerase, buffer, and dNTPs were from either Perkin–Elmer-Cetus or Promega. Superscript reverse transcriptase, RNasin, and RNase-free DNase were also from Promega. The panel of DNA from human–hamster somatic cell hybrids was purchased from Bios Inc. All other reagents were from Sigma.

Isolation of genomic clones. A human genomic DNA library was screened by a PCR method adapted from Green and Olson (1990). The library was primarily cultured to yield 50 phage cultures (each containing approximately 20,000 plaques per culture); the phage were then eluted from individual plates and centrifuged to remove cellular debris. A 5-μl sample from each of the 50 pools of phage was heat-denatured and then used for PCR. Primers for the PCR reaction were derived from the human NOS3 cDNA sequence (Janssens *et al.*, 1992; Marsden *et al.*, 1992); LR5, 5′-ATGGGCAACTTGAAGAGCGTGGCCC-3′ (bases 1–25; see Fig. 1); and LR6, 5′-TGTGTTCTGGCGCTGGTGGGAGTAG-3′ (reverse complement of bases 133–157). Each 50-μl reaction contained 200 ng of each primer, 2.5 U *Taq* polymerase, 50 m*M* KCl, 10 m*M* Tris–HCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* each dNTP, and 5 μl of DMSO. NOS3 cDNA and total human genomic DNA served as positive controls, and λ phage DNA was a negative control. The reaction was run for 40 cycles at 94°C for 1.5 min, 68°C for 1.5 min, and 72°C for 1.5 min. PCR products were separated by 1.5% agarose gel electrophoresis, and Southern blots of the gels were probed with an oligonucleotide (bases 26–43) ^{32}P-labeled using T4 polynucleotide kinase (general molecular biological techniques follow standard protocols described in Ausubel *et al.* (1990) and Sambrook *et al.* (1989)). Phage from the pool producing the expected PCR product were again cultured (6 cultures of 5000 plaques/culture) and screened by plaque hybridization. Clones hybridizing to a ^{32}P random hexamer-labeled 158-bp human NOS3 cDNA fragment (bases 1–158) were plaque purified. Restriction enzyme fragments of the genomic DNA containing NOS3 sequence were identified by Southern blotting (using the same probe) and subcloned into pBluescript, and the nucleotide sequence was determined according to the manufacturer's protocol for the Sanger dideoxy methodology.

RNA isolation and analysis. Total cellular RNA was isolated by the guanidinium thiocyanate method from human umbilical vein endothelial cells (HUVEC), cultured as previously described (Kourembanas *et al.*, 1991), and used in passages 2 through 5. For primer extension analysis, an oligonucleotide complementary to bases 6–35 of NOS3 was synthesized and end-labeled with ^{32}P using T4 polynucleotide kinase. The labeled primer (1×10^5 cpm) was hybridized to 20 μg of HUVEC RNA at 65°C for 90 min and extended for 1 h at 42°C using 200 units of reverse transcriptase according to the manufacturer's protocol. The reaction products were separated on a denaturing 8% polyacrylamide gel. A DNA sequencing ladder was used as a size marker. For use as a positive control, RNA was transcribed *in vitro* from a subcloned *Pst*I–*Sty*I fragment of the genomic DNA (bases −292 to +70, Fig. 1) using the Stratagene *in vitro* transcription kit according to the manufacturer's instructions.

RNase protection assays were performed essentially as described in Sambrook *et al.* (1989). A ^{32}P-labeled antisense RNA probe was synthesized from the same *Pst*I–*Sty*I fragment of the genomic DNA subcloned into pBluescript. This probe was hybridized to 10 μg of HUVEC RNA in 50% formamide at 65°C for 15 h. The samples were then digested using 2 μg/ml RNAse T1 and 40 μg/ml RNAse A for 1 h at 30°C, and the products were separated on a denaturing 8% polyacrylamide gel; a DNA sequencing ladder was used as a size marker.

Chromosomal localization. A panel of genomic DNA from 25 human–hamster somatic cell hybrids and from control human and hamster cell lines was screened by PCR. The sense primer (LR8) was 5′-CCTTCCGTCTGGTGCCACATCACAG-3′ (bases −379 to −355 of the NOS3 gene, Fig. 1). The antisense primer was LR6, described above. The PCR reaction was carried out as before, except that 1 μg of each primer was used either with 300 ng of somatic cell hybrid DNA or with 100 ng of the human or hamster control DNA. The reaction was run for 35 cycles consisting of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min. The PCR products were analyzed by agarose gel elec-

trophoresis and Southern blotting using the 158-bp cDNA probe as described above.

Fluorescence in situ hybridization. One microgram of a 20-kb human genomic clone containing NOS3 (λ44) was labeled with biotin-11-dUTP using a BioNick Labeling System (GIBCO BRL, Bethesda, MD). DNA was coprecipitated with 5 μg C_0t-1 DNA (GIBCO BRL) and resuspended in 1× TE at 100 μg/ml.

Metaphase chromosome preparations from peripheral blood lymphocytes obtained from karyotypically normal human males were denatured in 2X SSC in 70% formamide at 70°C for 2 min prior to dehydration in 70, 80, 90, and 100% ethanol at room temperature for 2 min each. Three microliters of biotin-labeled NOS3 probe was resuspended in 27 μl Hybrisol VI (Oncor, Gaithersburg, MD) and denatured at 70°C for 5 min. Fifteen microliters of denatured probe was applied to denatured chromosome preparations, a 24 × 50 mm coverslip was placed over the chromosomes, and slides were incubated in a humid chamber at 37°C overnight. Posthybridization washes included one wash for 15 min in 2X SSC in 50% formamide at 42°C, two washes for 5 min each in 2X SSC at 42°C, and one wash in 1X PBD (Oncor) for 5 min at room temperature. Biotin-labeled probe was detected using reagents supplied in the Oncor Kit and signal amplified according to manufacturer's recommendations. Metaphase chromosomes were counterstained simultaneously with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI) and propidium iodide (PI) solutions according to the protocol supplied with the Whole Chromosome Painting System (GIBCO BRL). Hybridization was observed using a Zeiss Axiophot microscope, and photomicrographs were taken with Kodak Technical Pan film at ASA 200.

Map position of the NOS3 probe was assigned by visual inspection of the fluorescent signal on the PI-stained metaphase chromosomes; chromosome identification was determined by DAPI staining. Twenty-seven metaphases were assessed for probe localization.

RESULTS AND DISCUSSION

Isolation and Characterization of an Endothelial NO Synthase Genomic Clone

A PCR-based screening approach was used to isolate genomic clones of the human endothelial NO synthase. We designed oligonucleotide primers based on sequences from the 5′ end of the human endothelial NO synthase cDNA to identify regions of the gene having potential regulatory significance. In the absence of intervening introns, these primers were predicted to yield a 157-bp product from genomic DNA as well as cDNA. This was confirmed, and the optimal PCR conditions were determined, in PCR experiments using these primers with samples of total human genomic DNA as template. We plated a human genomic DNA library in λFIX-II (Stratagene) to yield 50 pools, each containing 20,000 clones. PCR screening was then performed using DNA isolated from these pools, and the amplified products were resolved by agarose gel electrophoresis. One DNA pool (λ44) from the genomic library produced the expected PCR product, and individual positive clones from this pool were identified by plaque hybridization using a fragment of the NOS3 cDNA as a probe. One of these clones was plaque-purified, and its 20-kb insert was further characterized. Analysis of this genomic clone by restriction enzyme mapping and Southern blots, using the endothelial NO synthase cDNA as a probe, indicated that the NOS3 gene contains multiple introns and spans more than 20 kb (data not shown).

We then examined the 5′ end of the NOS3 gene, as represented in this genomic clone. Southern blot analysis of the clone identified a 6-kb *Not*I–*Hin*dIII fragment containing this 5′ region, which was then subcloned. Nucleotide sequence analysis of the subclone revealed the first coding exon of the NOS3 gene (Fig. 1). The genomic sequence of this exon is identical to the corresponding coding sequence of the cDNA (Janssens *et al.*, 1992; Marsden *et al.*, 1992); numbering is relative to the first base of the translation initiation methionine codon, with negative numbers referring to upstream sequences, counted in a 3′ to 5′ direction (see Fig. 1). The genomic sequence at the apparent exon/intron boundary conforms to an established consensus sequence (Breathnach and Chambon, 1981) for exon splice sites (Fig. 1). Upstream from the translation initiation site, the cDNA sequence we previously reported (Marsden *et al.*, 1992) is again identical to the genomic sequence, excepting only the first base of the cDNA. The other published sequence for the human NOS3 cDNA (Janssens *et al.*, 1992) extends upstream for a further 24 bases that show no significant similarity to the genomic clone. There are several possible explanations for this discordancy. An additional untranslated exon 5′ to the first coding exon might be included in some transcripts but not others, perhaps as a result of alternative splicing. However, the absence of a consensus sequence for an intron/exon boundary at this site in the genomic DNA argues strongly against this possibility. Alternatively, different cDNA sequences could reflect sequence polymorphisms of the NOS3 gene, error in the reported cDNA sequences, or cloning artifacts.

Identification of the Transcription Start Site

We have used several complementary experimental approaches to delineate the location of the 5′ end of the cDNA more clearly and to permit identification of the promoter region of this gene. Previous experiments using RACE (rapid amplification of cDNA ends), initially performed to isolate clones encoding the 5′ end of the cDNA (Marsden *et al.*, 1992), identified a possible transcription start site at −23. In these experiments, DNA was produced by reverse transcription (using a specific antisense primer derived from the cDNA sequence) followed by PCR amplification of the 5′ end of the mRNA, the product theoretically extending upstream as far as the transcription start site. The nucleotide sequence of the resultant cDNA contained 23 bases upstream from the translation start site, thus presumptively placing the transcription initiation site at −23. However, reverse transcriptase may stop prematurely in regions of unfavorable RNA secondary structure and thus identify a false transcription start site downstream from its true position. We therefore used RNase protection assays as well as primer extension analysis to confirm this assignment of the transcription start site.

We performed RNase protection studies next, as unfavorable RNA secondary structure should not affect

FIG. 1. Nucleotide sequence of the 5′ end of the human NOS3 gene. The sequence is numbered relative to the translation start site with negative numbers indicating sequence 5′ to the initiation ATG. The initiation codon is underlined and in boldface. The splice site consensus sequence at the first exon/intron boundary is underlined, and the end of the exon is marked with an arrow. The apparent transcription start site, as determined by primer extension and RNase protection assays, is also marked with an arrow and labeled. Consensus sequences for putative promoter and enhancer elements, including SP-1, GATA, AP-1, γ-IRE, and SSRE, are underlined and labeled. Both putative AP-1 sites overlap γ-IRE consensus sequences as indicated. *Pst*I and *Sty*I sites are in boldface.

these results as it does RACE and primer extension (Sambrook *et al.*, 1989). A radiolabeled antisense riboprobe was transcribed *in vitro* from a *Pst*I–*Sty*I fragment of the NOS3 gene (bases −292 to +70, Fig. 1). After hybridization to total cellular RNA from HUVEC and digestion by RNases A and T1, a single protected fragment was obtained (Fig. 2). The size of this fragment, by comparison with a DNA sequencing ladder, was estimated to be ~90 bp, placing the 5′ border of the first coding exon at or near the putative transcription start site identified by RACE.

Complementary experiments using primer extension then located the transcription initiation site more precisely. We selected an oligonucleotide primer (complementary to bases 6–35) binding within 100 bases of the putative transcription start site, since the probability of premature termination of reverse transcription increases with the distance of the primer from the transcription start site (the primer used for RACE was located ~400 bases downstream). First, RNA was transcribed *in vitro* (in the "sense" orientation) from the same *Pst*I–*Sty*I fragment of the NOS3 genomic clone and used as a positive control. Primer extension experiments using this RNA and the primer at +35 generated the expected ~370 base transcript. This demonstrated that the secondary structure of RNA near the putative transcription start site is not in itself an impediment to reverse transcription. From HUVEC RNA, a single major extension product was obtained in these experiments (data not shown); its size placed the transcription start site at −22, 1 base short of the site identified by RACE. However, the addition of a single nucleotide to the 5′ end of cDNA obtained by RACE has been observed previously (Frohman, 1990) and could explain this single base discrepancy.

Sequence Analysis of the Putative NOS3 Promoter

We determined the nucleotide sequence of ~2 kb of 5′ flanking sequence from the NOS3 gene to permit analysis of the putative promoter region. The sequence has been deposited with GenBank under Accession No. L23210. We found no TATA box (Breathnach and Chambon, 1981) near the transcription start site at −22. Although a TATA consensus sequence is found further upstream at −1964 (Fig. 1), at this location it is unlikely

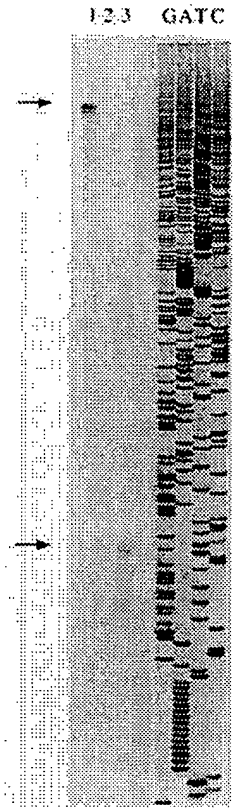

FIG. 2. RNase protection analysis of the NOS3 gene. An antisense RNA probe, complementary to bases −292 to +66 of the NOS3 gene, was hybridized to tRNA (lane 1), hybridized to tRNA and digested with RNase A and T1 (lane 2), or hybridized to HUVEC RNA and digested with RNase A and T1 (lane 3). Arrows indicate the undigested probe and the protected fragment from HUVEC RNA. A DNA sequencing ladder, using as primer a sense oligonucleotide at −1861, is included as a size standard.

to act as a promoter element for the NOS3 gene (Breathnach and Chambon, 1981). By contrast, a TATA box is present in the promoter of the inducible macrophage NO synthase gene (Xie *et al.*, 1993). The absence of a TATA box, however, is not unusual for the promoters of constitutively expressed genes (for review, see Ye *et al.*, 1993). A possible SP-1 binding site (a GC box; Dynan and Tjian, 1983) is present at −125 (Fig. 1) and could function as the major promoter element for the NOS3 gene. The reverse complement of a second GC box consensus sequence is found at −1353. The consensus sequence for another possible promoter element, a CAAT box (Benoist *et al.*, 1980), and its reverse complement are present at −312 and −654, respectively. The physiological significance of these sequences will be explored in planned functional studies, using reporter gene constructs designed to analyze the promoter region.

There are a number of *cis*-acting enhancer elements in the 5′ flanking region of the NOS3 gene that may mediate its observed regulation by shear stress and by inflammatory agents. Experiments *in vivo* and *in vitro* have demonstrated an increase in endothelial NOS activity in response to increased shear stress (reviewed in Furchgott and Vanhoutte, 1989; Moncada *et al.*, 1991). The increase in NOS activity is accompanied by an increase in NOS3 mRNA (Nishida *et al.*, 1992), which may reflect induction of transcription. A consensus sequence has been identified in the 5′ flanking region of several genes known to be induced by shear stress, and deletion of the identified consensus sequence, 5′-GAGACC-3′, in the PDGF gene blocks induction of PDGF by shear stress (Resnick *et al.*, 1993). The shear stress-response element is also found in the NOS3 gene at −2009, and at −1021, its reverse complement. These sequences may mediate enhanced NOS3 transcription and thus the increase in NOS3 activity in response to shear stress, a potentially important mechanism for blood pressure homeostasis.

Levels of NOS3 mRNA also appear to be regulated by inflammatory mediators (Lamas *et al.*, 1992). We have previously shown that, unlike macrophage NOS gene expression, which is induced by inflammatory mediators (Xie *et al.*, 1993), NOS3 mRNA levels decrease in response to cytokines such as TNF-α (Lamas *et al.*, 1992). The 5′ flanking region of the NOS3 gene lacks many of the canonical cytokine response elements found in the macrophage NOS gene. However, there are two possible AP-1 binding sites (Angel *et al.*, 1987; Lee *et al.*, 1987) in the NOS3 gene, at −684 and −1554. These could mediate down-regulation of NOS3 transcription in response to TNF-α, as has been described for other genes, such as elastin (Kahari *et al.*, 1992). Although several γ-IRE motifs are present in the NOS3 5′ flanking region, none is near an X box or a W element to permit gene induction in response to γ-interferon (Yang *et al.*, 1990; Pearse *et al.*, 1991). A GATA consensus sequence, found in other endothelial-specific genes (Wilson *et al.*, 1990), is also found in the NOS3 promoter region.

Chromosomal Localization of the Human NOS3 Gene

To identify the human chromosome containing the NOS3 gene, we used PCR to screen a panel of DNA from human–hamster somatic cell hybrids that contains different but overlapping sets of human chromosomes. The antisense (downstream) oligonucleotide primer was the same cDNA-derived primer (LR6) used for PCR screening of the genomic library. However, the sense (upstream) PCR primer was selected from the 5′ flanking region of the NOS3 gene. With these primers, a single amplification product of the expected size (536 bp) was obtained from PCR using total human genomic DNA as the template, but no product was obtained from hamster genomic DNA. The results of the PCR analysis of DNA from 25 human–hamster hybrid cell lines are summarized in Table 1. A PCR product of the correct size was amplified from only two somatic cell hybrids (none of the other hybrid cell lines generated any PCR products). The identity of the PCR product was confirmed by Southern blotting using the 5′ end of the NOS3 cDNA as probe (data not shown). As shown in Table 1, amplification of the correct PCR product segregated with chro-

TABLE 1

Mapping of the Human NOS3 Gene with Human–Hamster Somatic Cell Hybrids

		Human chromosome																						
Hybrid	NOS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
212	−					d																			+
324	−																		+						
423	−			+																					
507	−			+		+							+		d						d		d		+
683	−					+							d		+					+		+	+		
734	−					+				+									+						
750	−					d								+	+	+				+					
756	+					d	d	+					+	d	d					+	+	+			+
803	−				+	+			+			d											+	+	
811	−								+									d	d						
852	−		+																						
860	−			d		+	+				d									d		+			
862	−					+				+															
867	−	d				+								+	+				+	+					
904	−					d	+						+				d					+			+
909	−					d	+		+						+									+	
937	−	+				+									+			+				+			
940	−					+															+				
967	−					+			+								+								
968	−					+				+				+										d	
983	−					+					d														
1006	+				d	+		+	+					+		+				+		+			
1049	−					+							+												
1079	−			+		+										d	+								
1099	−	d				d								+						d		+	+		
% discordance		13	12	21	8	73	17	0	20	20	8	13	8	17	26	8	17	13	17	17	8	20	21	17	16

Note. The first column lists the individual hybrid somatic cell lines. The second column shows the results of PCR amplification of individual somatic cell hybrid DNAs; positive cell lines for the NOS3 PCR product are marked "+". Subsequent columns correspond to human chromosomes. Within these columns, a "+" indicates that the chromosome is fully represented in the corresponding somatic cell hybrid, and a "d" indicates that only a fragment or a deleted copy of that chromosome is retained. The final row reflects the % discordancy between presence of the individual chromosome and production of the expected product by PCR. Chromosomal assignment to human chromosome 7 is based on 0% discordancy with this chromosome.

mosome 7 with 0% discordancy. All other chromosomes were discordant with the PCR results for at least two somatic cell hybrids. These results permit the assignment of the endothelial NO synthase gene to human chromosome 7.

Localization of the NOS3 gene to chromosome 7 demonstrates that the two known constitutive NOS genes, like the NOS gene family as a whole, are not clustered on a single chromosome. However, the gene for a related enzyme, cytochrome P450 reductase, is present on chromosome 7 (Shephard *et al.*, 1989; Yamano *et al.*, 1990). Cytochrome P450 reductase resembles the C-terminal half of NOS and contains the same conserved cofactor binding sites for FMN, FAD, and NADPH. To determine whether the NOS3 gene might be near the gene for cytochrome P450 reductase, perhaps within a cluster of P450 reductase-related genes, we used FISH (Lichter *et al.*, 1990) to sublocalize the NOS3 gene on chromosome 7. Map position was determined by visual inspection of the fluorescence hybridization signals on PI-stained metaphase chromosomes coupled with chromosome identification by DAPI staining (Fig. 3). In 23 of 27 metaphase preparations analyzed, hybridization signal was found to be present in the telomeric region of the long arm of chromosome 7 corresponding to band q36; in 19 metaphases, both chromatids of both copies of chromosome 7 were labeled; and in 4 metaphases, signal was detected on both chromatids of one chromosome 7. Thus, *in situ* hybridization confirmed the assignment of the NOS3 gene to chromosome 7 and sublocalized it to 7q36. A polymorphic marker, D7S22 (Wong *et al.*, 1987; Royle *et al.*, 1988), is also found in this region and might prove useful in linkage studies of familial cardiovascular disease in which aberrant NO production is implicated.

Localization of the NOS3 gene to 7q36 clearly demonstrates that it is not clustered with the cytochrome P450 reductase gene, which is located at 7q11.2 (Shephard *et al.*, 1989; Yamano *et al.*, 1990). Nevertheless, it remains plausible that the progenitor NOS gene was formed by fusion of a gene resembling the N-terminus of NOS with one related to the cytochrome P450 reductase gene. Interestingly, several other proteins appear to contain a C-terminal cytochrome P450 reductase domain joined to an N-terminal domain derived from another protein family. For example, the *Bacillus megaterium* P450 monooxygenase is composed of a N-terminal cytochrome P450 and a C-terminal P450 reductase (Fulco, 1991). Another example is yeast flavohemoglobin, which

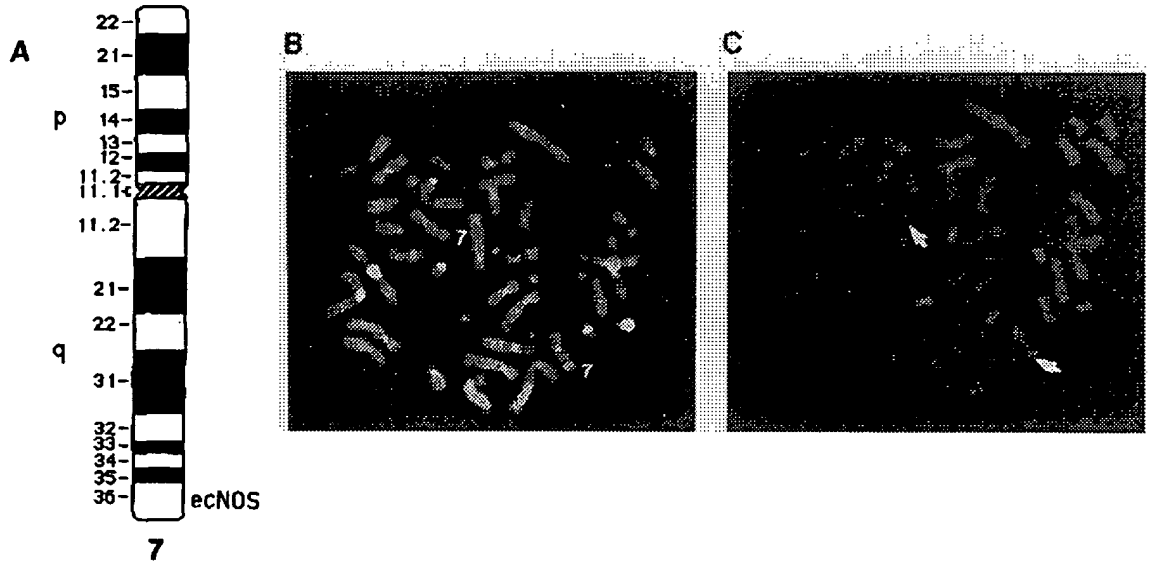

FIG. 3. Chromosomal localization of the NOS3 gene by fluorescence *in situ* hybridization (FISH). (A) Idiogram of human chromosome 7 showing the map location of the human NOS3 gene at 7q36. (B) Photograph of human metaphase chromosomes counterstained with DAPI. The two chromosomes 7 are indicated. (C) Photograph of human metaphase chromosomes counterstained with propidium iodide following FISH with a probe to the human NOS3 gene. Arrows point to the sites of hybridization on both chromosome 7 homologs in band q36; both chromatids show hybridization at that site.

has an N-terminal domain related to the globin family (Zhu and Riggs, 1992) and a P450 reductase domain at the C-terminus. However, the origin of the N-terminal half of NOS3 remains obscure. Although several NOS isoforms have been shown to contain a heme group with the spectrophotometric properties of a P450 cytochrome (reviewed by Marletta, 1993), none show sequence similarity to any member of the cytochrome P450 superfamily. Indeed, the N-terminal half of NOS3 bears no resemblance to any other known protein, aside from other NOS isoforms.

In summary, we have isolated a genomic clone encoding the human endothelial NO synthase gene, NOS3; identified its transcription initiation site; and determined the nucleotide sequence of its putative promoter region. This promoter region contains a GC box but no TATA box and thus resembles the promoters of other constitutively expressed genes. In addition, we identified possible *cis*-acting regulatory elements that may mediate changes in NOS3 transcription, and ultimately NO production, in response to physiologic stimuli such as shear stress and pathophysiologic stimuli such as TNF-α. We localized the NOS3 gene to chromosome 7q36, thus demonstrating that it is on the same chromosome but not adjacent to the gene for cytochrome P450 reductase from which it may have arisen. Ongoing efforts to characterize the NOS3 gene, and the structure-function relationships of the NO synthase protein, will provide further insight into the evolutionary mechanisms that can create a complex and novel enzyme.

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Note added in proof. Since the submission of this report, another research group (Marsden *et al.*, 1993) has described features of NOS3, and their findings are consistent with the data reported herein.

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